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Selecting *Bacillus* spp. Antagonist of Fungal Phytopathogen *Phytophthora infestans* Causing Tomato Late Blight

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Authors' contributions

This work was carried out in collaboration among all authors. Authors TLVK and LNT designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors MLT and MPT managed the analyses of the study. Author TLH managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The late blight caused by phytopathogen *Phytophthora infestans* has been one of the serious disease-causing yields and quality losses of tomato production in Vietnam. To control the fungal disease, chemical fungicides have been overused causing concerns about the ecological risks and human health, especially fungal resistance. Therefore, using natural products from beneficial microorganisms as a safer strategy is getting attention. The present study focused on the isolation of indigenous *Bacillus* sp. with potential antifungal activity against *Phytophthora infestans* with the aim to contribute to the diversification and improving the quality of biological control products *Bacillus* spp. in Vietnam. From 21 strains *Bacillus* spp. (marked BV1 - BV21) being isolated from different tomato farms in Danang City, Vietnam, *Bacillus velezensis* BV 16 was selected based on the most potential antagonistic strain in controlling fungal plant pathogen *Phytophthora infestans* attacking the tomato tree. The strongest inhibition of mycelial growth on P. infestans of *Bacillus*

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velezensis BV16 was recorded with 88.89% of growth inhibition percentage. The results also showed that strong activity of chitinase, protease and cellulase in the BV16 strain are consistent with the novel growth control of *Phytophthora infestans*.

Keywords: Fungal disease; plant pathogen; antifungal activity; biological control.

1. INTRODUCTION

Tomato (*Solanum lycopersicum*) has been recognized as an important crop in many countries of the world based on high nutritional value and economic efficiency. Recently, Vietnam agricultural sector has focused on investment and development to improve productivity and quality of tomato. However, pathogenic diseases have been destructing the crop, in which, the late blight caused by phytopathogen *Phytophthora infestans* has been one of the serious disease-causing yields and quality losses of tomato production that can reduce yield by 40-70% of total tomato production. *Phytophthora* is an oomycete, not a true fungus, which produces zoospores, therefore control of *Phytophthora* pathogens differs from others by true fungi and different fungicides are used [1]. One of the major control measures for *Phytophthora infestans* is agrochemical applications, for example, phosphonate, which bring fast efficiency, unfortunately, resistance populations of *Phytophthora infestans* fungicide drugs have developed [2]. Furthermore, if excessive and unsystematic applications, agrichemicals become an obstacle to sustainable agriculture and pose threat to the environment and humans alike [3,4,5]. Several nations have enacted policies to regulate the usage volume and types of agriculture chemicals [6,7]. Therefore, finding alternative approaches are needed for effective crop protection against this pathogen.

One approach that has been receiving much attention is biological control using antagonists such as *Trichoderma* and *Gliocladium*, mycoparasitic *Pythium* spp., nonpathogenic *Fusarium* spp., binucleate *Rhizoctonia* spp., *Laestisaria* spp., *Bacillus* spp., fluorescent *Pseudomonads* and *Streptomycetes* [8]. The usage of these microbial biocontrol agents would not only bring long-term economic benefits but also contribute to sustainable and effective organic agriculture development because of their potential characteristics such as producing antimicrobial metabolites including lytic enzymes, antibiotics, cyanide as well as plant-growth promotion compounds [9].

Some bacteria strains such as *Burkholderia, Pseudomonas, Bacillus* with novel antagonistic activity have been received much attention because they produce a wide range of extracellular metabolites that can inhibit and control fungal plant pathogens [10]. *Bacillus* spp. is considered to be an expert among the most promising antagonistic bacteria strains. In the research of Han at al. [10], five bacterial strains LB01, LB14, HM03, HM17, and LB15, were characterized as having antifungal properties in the presence of *Colletotrichum acutatum* and C. *gloeosporioides* in South Korea, two important antagonistic traits, siderophore production and solubilization of insoluble phosphate, were observed in the three *B. atrophaeus* strains. Analyses of disease suppression revealed that LB14 was most effective for suppressing the incidence of anthracnose symptoms on pepper fruits. LB14 produced antagonistic compounds and suppressed conidial germination of *C. acutatum* and *C. gloeosporioides*.

To contribute to the diversification and improving the quality of biological control products *Bacillus* spp. in Vietnam, the present study was carried out to screen antagonistic bacterial strains *Bacillus* spp. from tomato plants based on measuring suppression of growth of *Phytophthora infestans*, select the strongest antagonistic strain and determine some biochemical properties of the selected isolate.

2. MATERIALS AND METHODS

2.1 Collecting Samples

Soil samples were collected from the tomato roots of both diseased and non-diseased plants, placed in the sterile polypropylene bags and kept at 4^{o}C, then transported to Laboratory for isolation of bacteria. Every separated bag containing 100g of soil was recorded with name, date and location

2.2 Isolation of Bacteria

The collected soil sample was serially diluted by solution NaCl of 0.9% up to 10^{-7}. Heating in a water bath at 80^{o}C for 10 minutes was conducted to remove vegetative cells and other

microorganisms. A drop of 100 µL of dilution was spread on nutrient agar plate Luria-Bertani (LB) medium. The plates were incubated at 37°C for 24 h. The particular colonies of *Bacillus* sp. were selected and sub-cultured in on nutrient agar plate until achieving pure colonies. The pure colonies were stored on nutrient agar slants (LB medium) at 4°C. The identification of the genus was based on the morphology, gram staining and biochemical test of *Bacillus* sp.

2.3 Antifungal Activity Assay

Antifungal activity assay was imposed on a PDA plate with a center hole containing the fungal pathogen *Phytophthora infestans* and other holes at corners containing a volume of 20 µL of bacteria overnight inoculate. All of the cultures were incubated at 30°C. The growth of fungal pathogens was evaluated 7 days after incubation. Inhibition of fungal growth was measured as the distance of the clear zone between the bacterial colony and each fungus according to the following formula [10]:

$$I = \frac{(R - r)100}{R}\%$$

In which, I was inhibition of mycelial growth (%), R and r were the distance between the center and fungal hyphae edges in the control and treatment plates, respectively.

2.4 Identification of Selected Strain Based on 16S Rrna Gene Sequencing

DNA was extracted from the bacterial cells using a Wizard genomic DNA purification kit (Promega, USA). The 16S rRNA was amplified with the universal primers 27F (AGAGTTTGATCMTGGCTCAG), 1492R (TACGGYTACCTTGTTACGACTT), in Thermal Cycler Thermal Controller 2720 (Applied Biosystems, USA). PCR product was separated on gel agarose 1% and purified using GeneJET GelExtraction Kit (Thermo Scientific, USA). Sequencing of 16S rRNA gene was performed by the Sanger's method, then using BLAST NCBI +analysis to find the match sequence. The phylogenetic relationship was constructed by MEGA X software using the nearest-neighbor data analysis method with 1000 bootstrap replicates.

2.5 Extracellular Enzyme Assay of Selected Strain

Chitinase activity was carried on YEG (Yeast extract glucose) medium agar (4 g/L yeast extract, 20 g/L glucose, 20 g/L agar) adding 1% of colloidal chitin [11]. Chitin degrading ability was detected by development of a clear zone after incubation for 3 days at 25°C.

Screening for extracellular cellulase production was carried out on agar plates containing CMC as substrate ((1.0 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.5 g/L NaCl, 0.01 g/L $FeSO_4 \cdot 7H_2O$, 0.01 g/L $MnSO_4 \cdot H_2O$, 0.3 g NH_4NO_3, 10 g/L carboxymethyl cellulose, 12 g/L agar) media [12]. After incubation at 25 °C for 3 days, the agar was flooded with 0.1% Congo red for 15 min to 20 min, and then with 1 M NaCl for 15 min to 20 min. The detection of the cellulolytic activity in these cases was achieved by staining of undigested CMC in plate regions which was not exposed to cellulolytic activity, while areas exposed to cellulase give clear halos surrounding the source of the enzyme.

Protease production of the bacterial strain was screened on agar plates supplemented with 5% NaCl and 1% casein (MNA). The plate was incubated overnight at 37 °C. The protease producing strains were selected based on the zone of clearance [4].

2.6 Statistical Analysis

All experiments were performed in triplicate. The obtained results were shown as the average and standard deviation (SD). The excel software (2010) was used to analyze the obtained data. The significant test was set at $p \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1 Screening Antifungal Activity of Isolates

In the present study, from soil samples of three tomato farms in Danang City, Vietnam, 21 strains were identified to have the emblematic morphology and biochemical characteristics of *Bacillus* sp. marked BV1 – BV21 (Table 1).

To selecting the isolate having the highest antagonistic capacity against *Phytophthora infestans* causing tomato late blight, antifungal activity assays of 21 isolates were carried ou. The obtained results showed that thirteen

bacteria strains could inhibit the growth of *P. infestans*, in which six strains exhibited high antifungal activity including BV2, BV7, BV8, BV10, BV16, BV21 (Fig. 1). The strain BV16 exerted the strongest inhibition of mycelial growth on *P. infestans,* making up 88.89% of growth inhibition percentage which was 18.89% higher compare to eighteen *Bacillus* spp. and seven *Pseudomonas* spp. in the research of Caulier et al. [13]. Therefore, BV16 was selected to further study.

3.2 Identification of Selected Strain Based on 16S Rrna Gene Sequencing

To identify strain BV16 with the highest inhibitory effects on the growth of *Phytophthora infestans*, the 16rRNA sequences were analyzed. The obtained result revealed that BV16 displayed 99% sequence similarity with its closest relative *Bacillus velezensis* FZB42 (Fig. 2, Fig. 3).

Table 1. Biochemical charaterization of isolated strains

Strain	Colony morphology	Cell shape	Gram staning	Spore	Mobility	Catalase	Oxidase	VP
BV1, BV3, BV17	Milky white, rounded, mucus, convex	Rod	+	+	+	+	+	+
BV2, BV4, BV13, BV12	Opaque white, smooth	Rod	+	+	+	+	+	+
BV5, BV6, BV14	Yellow, rough	Rod	+	+	+	+	+	+
BV7, BV15, BV19, BV20	Milky white, dry, wrinkled	Rod	+	+	+	+	+	+
BV8, BV9, BV11	Opaque yellow, smooth with mucus	Rod	+	+	+	+	+	+
BV10, BV18	Opaque white, dry, wrinkled border	Rod	+	+	+	+	+	+
BV16	White, large, rough and wrinkled border	Rod	+	+	+	+	+	+
BV21	White, wrinkled, convex	Rod	+	+	+	+	+	+

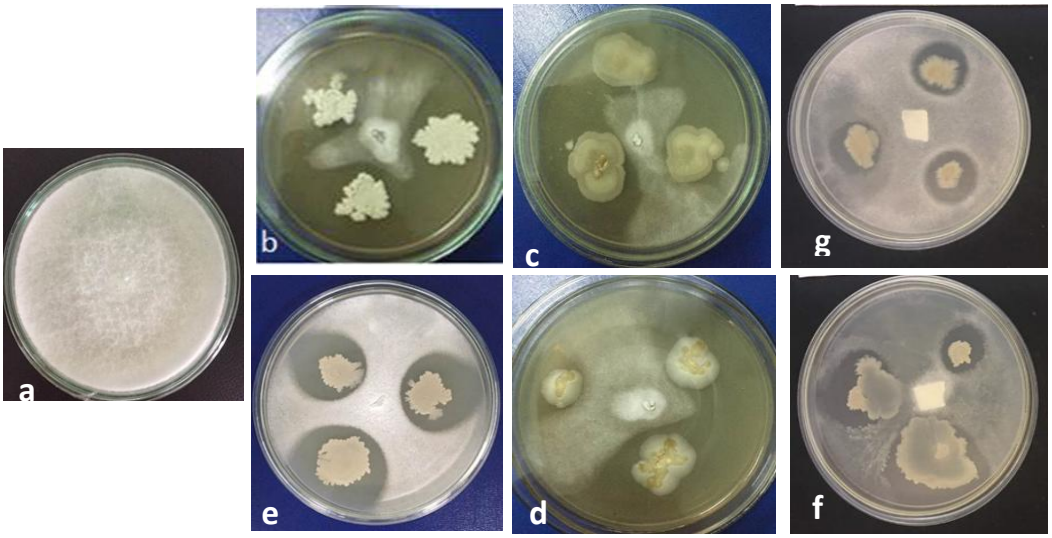

Fig. 1. Antifungal activity of isolated *Bacillus* strains
(a: Control, Antifungal activity of BV16 (b), BV21 (c), BV8 (f) , BV2 (g), BV10 (d), BV7 (e) against Phytophthora infestans)

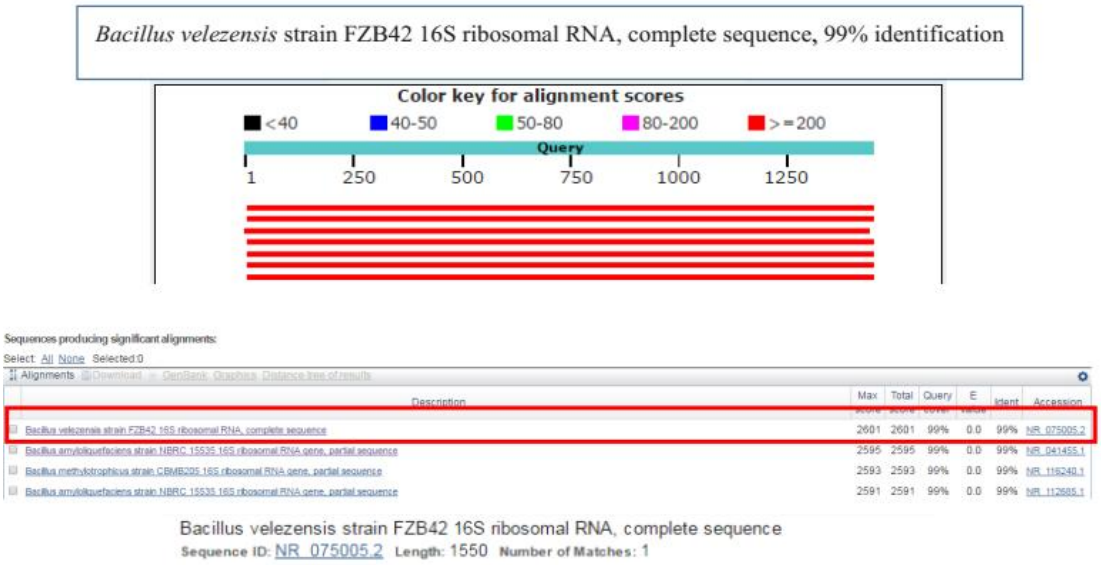

Fig. 2. The result of the 16rRNA sequences

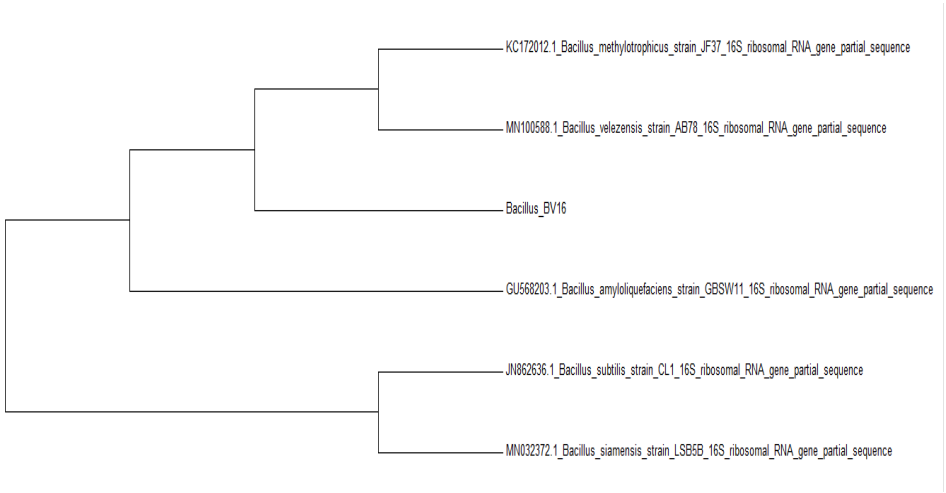

Fig. 3. Phylogenetic relationship based on 16rRNA sequences of BV16

Bacillus velezensis was described as gram-positive bacteria with rod-shaped, cell size from 0.7 - 0.9 × 1.6 - 3 µm, producing acids from aesculin, amygdalin, arbutin [14]. This bacteria was also known as an effective biocontrol agent of plant pathogen with promising ability to produce antibiotics, fungal cell wall – degrading enzymes and plant growth stimulating compounds [15]. The research of Fan et al. [16] summarized that the strain of *B. velezensis* FZB42 and mutants controlled plant pathogens due to different mechanisms including direct antibiosis and competition by secretion of a spectrum of secondary metabolites in the rhizosphere, the beneficial action on the host-plant microbiome and stimulation of plant induced systemic resistance (ISR). Importantly, the harmful effects of *Bacillus velezensis* to date, have not been reported yet.

3.3 Extracellular Enzyme Assay of Selected Strain

An enzyme produced by microorganisms is determined based on the ability to decompose substrates and change the color of the specific media around the bacterial colonies. The ability of chitinase, cellulase and protease – producing

ability of *B.velezensis* BV16 strain was screened and the obtained results were presented on Table 2 and Fig. 4.

Table 2. Diameter of clear halos surrounding extracellular enzymes

Strain	Diameter (cm)		
	Chitinase	Protease	Cellulase
BV16	2.1 ± 0.2	1.2 ± 0.2	2.5 ± 0.1

B. velezensis is capable of producing chitinase, protease and cellulase which contributed to fungal cell wall breakdown. The fungal cell wall is a matrix composed of polysaccharides, proteins, and other components, for example, many fungal pathogens belonged to ascomycota and basidiomycota contain mixture of chitin, glucan, and mannoproteins in their cell walls [17], that provide novel structural barriers in order to develop, survive in response to ambient conditions. Therefore, to exhibit antifungal activity, many antagonistic bacteria need to secrete lytic enzymes capable of dissolving fungal cell walls [18]. Especially, proteases also play a key role in the cell lysis process. Proteases bind to the outer mannoprotein layer of the cell wall, open the protein structure, and expose inner glucan layers and chitin microfibrils [10]. Strong activity of chitinase, protease and cellulase in the BV16 strain are therefore consistent with the observed novel growth inhibition of *Phytophthora infestans* (Fig. 1b).

3.4 Control Efficiency from Biomass, Extracellular Fluid, Extracellular Enzymes and Non–enzyme Metabolites Produced by Selected Strain against *Phytophthora infestans* after 7 days

The results of Table 3 and Fig. 5 showed that there was a significant difference in the inhibitory efficiency against fungal pathogen *Phytophthora infestans* from the different components of the selected strain *B. velezensis* culture broth including biomass, extracellular fluid, extracellular enzymes and non-enzyme metabolites.

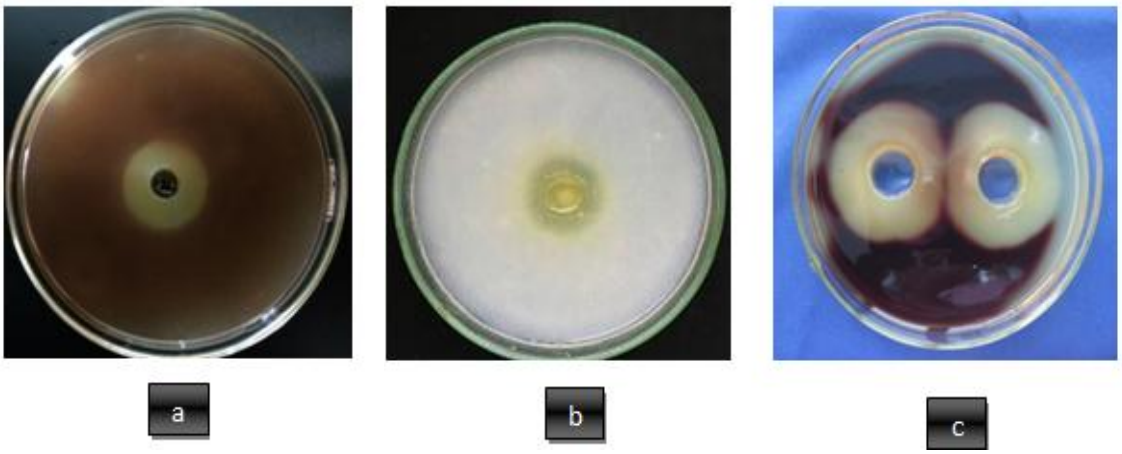

Fig. 4. Production of chitinase (a), protease (b), cellulase (c) by BV16 strain

Table 3. Control efficiency from different components of selected strain *B. velezensis* culture broth against *Phytophthora infestans* after 7 days

Treatment	Inhibition of mycelial growth (%)
Control (*Phytophthora infestans)*	-
Biomass (10^4 cells/mL)	**84.44±2.22[a]**
Biomass with heat - treantment (10^4 cells/mL)	66.67±3.84[b]
Extracellular fluid	**80.00±2.22[a]**
Extracellular enzymes	72.59±5.59[b]
Extracellular non-enzyme metabolites	45.18±3.39[c]

**Means ± SD = Means ± Standard Deviation of three independent experiences. The mean difference is significant at the p ≤ .05. Means followed by the different letters are significantly different*

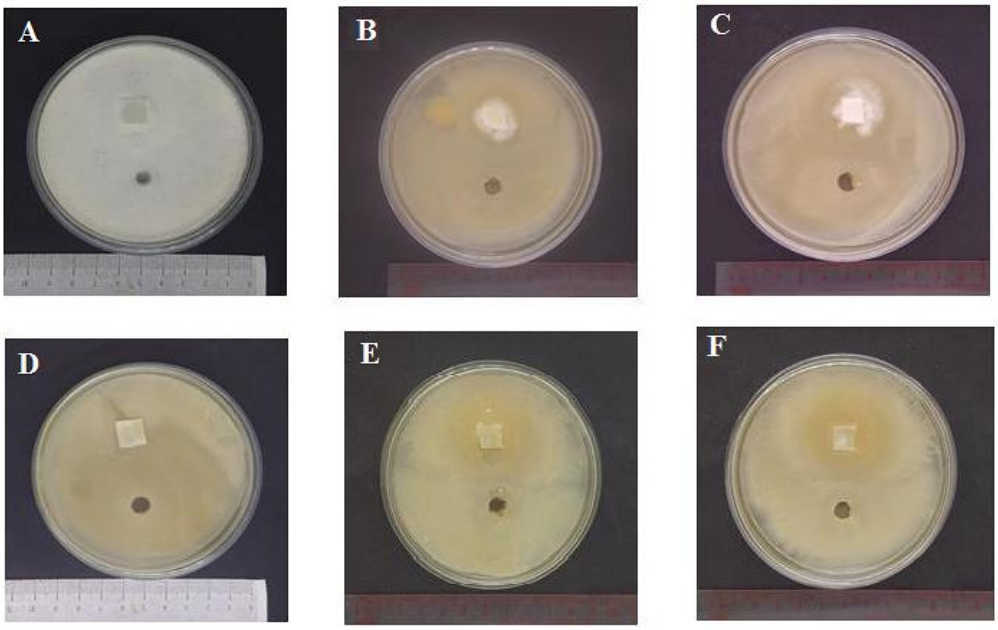

Fig. 5. Potential antifungals in the different components *B.velezensis* BV 16 culture broth*:* A. Control; B. Biomass; C. Biomass with heated up to 55°C; D. Extracellular fluid, E. Extracellular non-enzyme compounds, F. Extracellular enzyme

Among them, the highest inhibitory efficiency was observed at the biomass, making up 84.44%, while bacterial biomass with being heated up to 55°C also exhibited the antagonistic ability with 66.67% of inhibitory efficiency. This can be explained that *B. velezensis* cells could compete strongly nutrition with the fungal pathogen [19]. Besides, in unfavorable conditions (such as being heated), *B. velezensis* cells could produce spores and release internal metabolites resulting in inhibiting the growth of the fungal pathogen. Furthermore, the result also showed that extracellular fluid of *B. velezensis* BV16 was responsible for 80% of control efficiency against the fungal disease *Phytophthora infestans* on tomato crop. This finding was consistent with the previous researches also revealed that potential antifungal metabolites produced by *B. velezensis* could consist of lipopeptide, polyketide, dipeptide, siderophore, difficidin, bacillaene, macrolactin and antimicrobial protein [20,21,22]. Especially, the inhibition of mycelial growth of the extracellular enzymes was 1.6 times higher than the figure of extracellular non-enzyme compounds, which was in agreement with the novel capacity of producing some extracellular enzymes of *B. velezensis* BV16 including chitinase, protease and cellualse (Fig. 4).

4. CONCLUSION

The present study showed that BV16 was *Bacillus velezensis* which has known as the most potential antagonistic strain in controlling fungal plant pathogen *Phytophthora infestans* attacking the tomato tree. The strongest inhibition of mycelial growth on *P. infestans* of *Bacillus velezensis* BV16 was recorded with 88.89% of growth inhibition percentage. The results also showed that strong activity of chitinase, protease and cellulase in the BV16 strain are consistent with the novel growth control of *Phytophthora infestans*. This result will be applied to produce the biological control as the alternative method contributing significantly to the development of sustainable and efficient organic agriculture.

ETHICAL APPROVAL

This article followed professional ethics within its research. As per international standard or university standard written ethical approval has been collected and preserved by the author(s).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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